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ON THE NATURE OF THE CONDITIONS WHICH DETERMINE OR PREVENT THE ENTRANCE OF THE SPERMATOZOON INTO THE EGG

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I

THE well-known fact that a spermatozoon can no longer enter an egg after it is once fertilized raises the question whether this is due to the changes necessarily connected with development; or whether development of an egg can take place without the existence of such a block. We are in possession of facts speaking in favor of the second view. Thus the writer has shown that if the eggs of *Strongylocentrotus purpuratus* or *Arbacia* are induced to develop by the methods of artificial parthenogenesis a spermatozoon can enter the egg or an individual blastomere of a segmenting egg, while the latter is in the full process of development. This leaves no doubt that the block caused by the entrance of a spermatozoon into an egg for the entrance of further spermatozoa must be due to a change not necessarily identical with that inducing the development of the egg.

A second group of observations made by the author deals with the phenomena of specificity and these prove that the block which an egg offers to heterogeneous sperm is rapidly reversible and confined to the surface of the egg or the spermatozoon or both. In the case of the egg of *purpuratus* and sperm of *Asterias* (and many similar in-

stances) the specific block can be overcome if we slightly increase the alkalinity of the sea water. The spermatozoon can only enter the foreign egg while both sperm and egg are in the hyperalkaline sea water, whereas if the egg and sperm are treated separately with hyperalkaline solution (no matter how long) and put together in a sufficiently large quantity of normal sea water no egg can be fertilized[1] while fertilization will take place as soon as the hyperalkalinity is restored. This shows that the change (brought about by the hyperalkaline sea water) which makes the fertilization possible is rapidly reversible, as we should expect it to be if it consisted merely in a physical change at the surface of the egg. To this series of facts, others might be added which point in the same direction. In this paper we intend to discuss a little more fully the various conditions which block or favor the entrance of a spermatozoon into an egg, in order to form an idea of the nature of the forces which control these phenomena.

II

1. When the unfertilized eggs of *S. purpuratus* are treated for two hours with hypertonic sea water (50 c.c. sea water + 8 c.c. $2\frac{1}{2}$ *m* NaCl or Ringer solution) the eggs of certain females will develop into blastulæ, gastrulæ and plutei, while the eggs of other females can not be caused to develop in this way. These individual differences coincide possibly with those observed by the writer in regard to spontaneous membrane formation in the eggs of different females[2] and it is possible that only the eggs of such females of *purpuratus* can be induced to form larvæ through a mere treatment with hypertonic sea water in which the latter can induce the cortical changes underlying the membrane formation. Whatever the nature of the individual difference may be, *purpuratus* eggs

[1] The large quantity of sea water is necessary so that the hyperalkaline sea water at the surface of the egg and sperm can diffuse away before both gametes come in contact.

[2] Loeb, *Arch. f. Entwcklngsmech.*, XXXVI, 626, 1913; "Artificial Parthenogenesis and Fertilization," Chicago, 1913, p. 219.

which have been induced to develop into larvæ by a hypertonic solution can be fertilized with sperm while they are in the process of segmentation. When such eggs are in the two-, four-, eight-, or sixteen-cell (and possibly also later) stages the sperm can enter into one or more blastomeres of such an egg and this entrance betrays itself by a distinct and clear membrane formation around each blastomere.[3] While the segmenting eggs which were not fertilized with sperm develop into larvæ, those into which sperm enters perish very rapidly. This simple and rather striking experiment which can easily be performed in the eggs of *Strongylocentrotus,* where the membrane formation around a single blastomere can be clearly recognized, shows that the process of development in a fertilized egg in itself can not be responsible for the block caused by fertilization. It looks as if the entrance of a spermatozoon into the mature egg, independently of the developmental changes it induces in the egg, causes some physical or physico-chemical change (of the surface of the egg?) which renders the subsequent entrance of a spermatozoon impossible.

2. With the eggs of most females of *purpuratus* the treatment with a hypertonic solution does not lead to a development into larvæ, but only to the first segmentation stages in a limited number of eggs (provided that the eggs have been exposed to the solution the proper period of time). Such blastomeres afterwards go into a resting stage. If one waits long enough, until there is no doubt left that the blastomeres have reached a resting condition and will divide no further, and if one then adds sperm, the individual blastomeres can again be fertilized, which is indicated by a membrane formation around each individual blastomere and the subsequent development of such blastomeres into swimming larvæ.[4] The fact that each individual blastomere in this case is fertilized independ-

[3] Loeb, "Artificial Parthenogenesis and Fertilization," p. 240; *Arch. f. Entwcklngsmech.,* XXIII, 479, 1907.

[4] Loeb, *Arch. f. Entwcklngsmech.,* XXIII, 479, 1907; "Artificial Parthenogenesis and Fertilization," p. 237.

ently of its neighbors suggests that there is no protoplasmic connection between the neighboring blastomeres; otherwise the entrance of a spermatozoon into one should cause its neighbors also to form a fertilization membrane, which does not happen.

All these facts show that the changes underlying development do not necessarily prevent the entrance of a spermatozoon into an egg fertilized by sperm.

3. Development can be initiated in an unfertilized egg by causing a membrane formation by a fatty acid. Eggs after such an artificial membrane formation perish as a rule rapidly at room temperature (if no second treatment is given them) but they may segment if kept at a low temperature. The eggs are usually put after treatment with the butyric acid into normal sea water in which they form a membrane. This membrane is different in the eggs of different species of sea urchins. In the egg of *S. purpuratus* the membrane is tough and entirely impermeable to the spermatozoon. When we add sperm to such eggs with a butyric acid membrane they behave exactly as if no sperm had been added, they all perish rapidly (at room temperature). The question arose, if a spermatozoon could still enter the egg of *purpuratus* after membrane formation, provided the membrane could be destroyed. This can be done in a certain percentage of the eggs of *purpuratus* by shaking them after artificial membrane formation; the number of eggs whose membrane is torn varies in different experiments owing probably to differences in the thickness and toughness of the membrane. Even if the membrane is torn the edges may come close together again so that the opening often is closed again and no spermatozoon can go through. Kupelwieser and the writer performed this experiment on the eggs of *purpuratus* and it was found that such eggs with torn membranes were fertilized upon the addition of sperm and developed normally; while the eggs whose membranes were intact all perished.[5]

[5] Loeb, "Artificial Parthenogenesis and Fertilization," p. 234.

The writer repeated this experiment last winter with the same result. He found that eggs with torn membranes when subsequently fertilized with sperm did not form any new membranes as he had stated before. It is possible that he mistook at that time the new hyaline membrane which forms around the egg after membrane formation and fertilization for a new fertilization membrane.

It is not necessary that these eggs be fertilized immediately after the artificial membrane formation, the experiment succeeds also after some time (one hour or more); only with this difference that the eggs perish very rapidly after the membrane formation if they receive no second treatment. In order to avoid this difficulty the writer last winter proceeded as follows: Artificial membrane formation was produced in the eggs of a *purpuratus* and all eggs had formed perfect membranes. One control was kept and the rest were shaken. These were divided into three lots, one served as a control; the eggs of the latter all perished as fast as the eggs of the first control (which were not shaken). The second lot were fertilized after about one half hour after membrane formation. Twenty per cent. of these eggs developed into normal larvæ, the rest perished. The percentage of developing eggs corresponded roughly with the percentage of eggs whose membrane was torn. The third lot of the shaken eggs was put overnight into 50 c.c. sea water + 7 drops of 1/10 per cent. KCN, to prevent the disintegration of these eggs. The next morning (sixteen hours after the membrane formation) the eggs of Lot 3 were transferred into normal sea water and divided into two lots, one was fertilized with sperm, the other was kept as a control. About twenty per cent. of the eggs which were fertilized began to segment, but many in an abnormal way and none developed into larvæ. Of the second lot to which no sperm was added also a few began to segment. As the writer has shown in former experiments, the eggs of *Strongylocentrotus* can be caused to develop after artifi-

cial membrane formation if they are either treated for a short time with a hypertonic solution or if for a longer period the oxidations are suppressed in them by lack of oxygen or the addition of cyanide. There is therefore no doubt that the eggs of *purpuratus* in which the artificial membrane formation has been induced by butyric acid can be fertilized subsequently with sperm.

4. The treatment of the eggs of *Arbacia* with butyric acid leads to the formation of a membrane which varies considerably in the eggs of the same female. Some eggs have a thin membrane which is permeable to the spermatozoon, others have a tough fertilization membrane which is as impervious to the spermatozoon as the regular fertilization membrane. The percentage of the eggs with membranes permeable for sperm varies very much in different experiments, according to the material and according to the external conditions. If this is kept in mind it is easily understood that the number of *Arbacia* eggs which can be fertilized after they have been treated with butyric acid differs in different experiments. Since the membrane called forth by butyric acid is not always plainly visible, it is a prerequisite that always one set of such eggs should be set aside as controls to ascertain whether or not *all* the eggs disintegrate rapidly (if no second treatment is given to them). Only if they all disintegrate rapidly have we any guarantee that in all of them the membrane formation has been effective. The former experiments of the writer show that such eggs can be fertilized by sperm; in fact they show that while the unfertilized eggs disintegrate rapidly after the inducement of the membrane formation with butyric acid, the subsequent fertilization of such eggs by sperm saves their lives and makes them develop.[6]

III

1. It is a well-known fact that most eggs can only be fertilized by sperm of their own or a closely related species. The writer thought that in order to obtain light

[6] Loeb, *Arch. f. Entwcklngsmech.*, XXXVIII, 416, 1914.

on the nature of the block to the entrance of heterogeneous sperm it was necessary first to find the means by which this block could be overcome. He succeeded in showing that the egg of the sea urchin *S. purpuratus* can be fertilized by the sperm of starfish, brittle stars, and holothurians in sea water (or other balanced solutions) if their alkalinity was a trifle higher than that of ordinary sea water (*e. g.*, in a solution of 50 c.c. sea water + 0.6 c.c. *N*/10 NaOH).[7] Godlewski[8] succeeded by the same method in the fertilization of the egg of the sea urchin with the sperm of crinoids

The most important fact found out in this connection was the following, namely, that the fertilization of the egg of *purpuratus* by the sperm of *Asterias* only takes place while both eggs and sperm are in this hyperalkaline solution. If eggs and sperm are put into these solutions separately and if then from time to time sperm and eggs so treated are transferred into normal sea water, as a rule not a single egg is fertilized; while with the same material when eggs and sperm are together in the hyperalkaline solution as many as 100 per cent. of the eggs may be fertilized. The effect of the alkali is, therefore, rapidly reversible; the eggs when put from the hyperalkaline sea water free from sperm into the normal sea water containing very motile sperm of *Asterias* can not be fertilized; when put back into hyperalkaline sea water containing *Asterias* sperm they will be fertilized rapidly.

This rapid reversibility of the effect of the NaOH indicates that it must be confined to the surface of the egg and the spermatozoon or both; and this is corroborated by the fact that the NaOH does not enter the cells. One of the forces which determine the entrance of the spermatozoon into the egg may be surface tension and the phenomenon of the entrance may be comparable or possibly identical with the phenomenon of phagocytosis.

Godlewski mentioned that he occasionally observed a

[7] Loeb, *Pflüger's Arch.*, IC, 323, 1903; CIV, 325, 1904; *Arch. f. Entwcklngsmech.*, XL, 310, 1914; *Science*, N. S., XL, 316, 1914.

[8] Godlewski, *Arch. f. Entwcklngsmech.*, XX, 579, 1906.

fertilization of the egg of the sea urchin with the sperm of a crinoid in normal sea water after both had been treated with hyperalkaline sea water separately. This observation is correct but finds its explanation in the assumption that in such cases the hyperalkaline sea water had not had time to diffuse from the jelly of the egg or from the surface of the egg protoplasm by the time the spermatozoon came in contact with it. In order to test this view the writer treated the eggs of *purpuratus* with a hyperalkaline solution of greater than the optimal concentration while the sperm was treated separately with the optimal concentration (50 c.c. sea water + 0.6 c.c. $N/10$ NaOH) and then both were mixed in a little sea water in a watch glass. In such a case a large number of eggs were fertilized, but while the fertilization occurred nominally in normal sea water it really occurred in a layer of hyperalkaline sea water surrounding the protoplasm of the egg.

The conclusion from these experiments is that the block to the entrance of the spermatozoon of *Asterias* into the egg of *purpuratus* is of a rapidly reversible character, consisting in some alteration of a physical property of the surface. On this assumption the factor of specificity consists of an agency which affects these properties of the surface of the egg in the same sense as the increase in the concentration of the alkali. It should be added that the writer observed also that an increase of the concentration of Ca in the sea water acts in the same sense as an increase in the alkalinity; and that if the concentration of Ca is increased the increase of NaOH may be less than is necessary otherwise.

2. If the idea was correct that the factor of specificity contained in the spermatozoon affected only the forces acting at the surface of the egg; and that the lack of this factor could be replaced by a rise in the alkalinity of the sea water, it was to be expected that the reverse should also be possible: namely, that a change in alkalinity or the constitution of the surrounding medium should pro-

duce a reversible block to the spermatozoa of the same species. That means, it should be possible to find solutions in which the egg does not suffer for a long time, in which the sperm lives for a long time, and in which the sperm of the same species is intensely active and attacks the egg with the greatest eagerness and yet is not able to enter; while if the medium is but slightly changed the sperm enters the egg at once. The writer carried out such experiments a year ago in Pacific Grove and last summer in Woods Hole and found this to be true.[9]

For the purpose of these experiments the ovaries and testes of the sea urchins were not put into sea water but into a pure $m/2$ NaCl solution (after several washings in such a solution) and kept in such a solution. Several drops of sperm and one drop of eggs were in one experiment put into 2.5 c.c. of a neutral mixture of $m/2$ NaCl and $3/8\ m$ $MgCl_2$ in the proportion in which these two salts exist in the sea water. In such a neutral solution no egg of *Arbacia* or of *purpuratus* is fertilized no matter how long they remain in the solution, although the sperm is very active. If the eggs and sperm are transferred into the same solution which contains in addition 1 drop of a $N/100$ solution of NaOH (or NH_3, or benzylamine, or butylamine) or 8 drops of $m/100$ $NaHCO_3$, most and often practically all the eggs at once form fertilization membranes and begin to segment at the proper time.

The same result can be obtained if the eggs are transferred into a neutral mixture of $NaCl + MgCl_2 + CaCl_2$ (in the proportion in which these salts exist in the sea water) or into a neutral mixture of $NaCl + MgCl_2 + CaCl_2 + KCl$. In such a neutral mixture the eggs form fertilization membranes and begin to segment.

The eggs will not be fertilized if transferred into a neutral solution of NaCl or of $NaCl + KCl$.

It is, therefore, obvious that if we diminish the alkalinity of the solution surrounding the egg and if we deprive this solution of $CaCl_2$ we establish the same reversible

[9] Loeb, *Science*, N. S., XL, 316, 1914.

block to the entrance of the spermatozoon of *Arbacia* into the egg of the same species as exists for the entrance of the sperm of starfish into the egg of *purpuratus* in normal sea water.

Another form of the experiment may be mentioned. When we put sperm and eggs of *Arbacia* (which had been washed in an $m/2$ NaCl solution) into a neutral mixture of NaCl + KCl no egg can be fertilized although the sperm may be so active and concentrated that the eggs roll around in the solution and the chorion (the jelly surrounding the egg) may be filled with spermatozoa. In one experiment the eggs and sperm of *Arbacia* were kept overnight in watch glasses containing 2.5 c.c. of this mixture of neutral NaCl + KCl. The next morning all the eggs were intact and not a single one was fertilized. At that time 20 drops of sea water were added to the mixture and instantly fertilization membranes were formed and practically all the eggs segmented.[10]

It can be shown that in this experiment the sea water added two important substances, Ca and NaOH. If NaOH alone is added to the mixture of NaCl + KCl, as a rule no egg is fertilized or only a few; if $CaCl_2$ is added to a neutral mixture of NaCl + KCl a number of eggs are fertilized. If both $CaCl_2$ and NaOH are added in the proper proportion as a rule all the eggs are fertilized.

It is perhaps important to call attention to the fact that if eggs of *Arbacia* are fertilized in sea water and if after repeated washings in a mixture of NaCl + KCl or of NaCl + $MgCl_2$ they are put into these solutions they will segment repeatedly in these solutions, thus showing that the eggs were really not fertilized in these two solutions in the above-mentioned experiments.

The striking fact is again that the block created by the

[10] This experiment was carried out with different concentrations of sperm and it was found that only in the dishes where the concentration of sperm was sufficiently high were all the eggs fertilized upon the addition of sea water. This is perfectly natural as the majority of spermatozoa die gradually (as do also the eggs) and hence enough spermatozoa will only be alive the next day if the concentration of sperm was not too low.

lack of $CaCl_2$ or NaOH or both to the entrance of the spermatozoon is removed immediately after these substances are added. The block must be due merely to a change in the physical condition of the surface (which may be based on a rapidly reversible chemical reaction).

In these experiments the NaCl can not be replaced by isotonic sugar solutions. The same fact was found by the writer to be true for heterogeneous hybridization.

It is of importance to call attention to the fact that the abolition of the block in the case of heterogeneous hybridization depends upon the same substances, $CaCl_2$ and NaOH (or some other alkali), which make normal fertilization possible. The influence of electrolytes on the fertilization of the egg of *purpuratus* by the sperm of *Asterias* is parallel to the influence of the same electrolytes on the fertilization of the same egg by the sperm of *purpuratus;* only the concentrations differ, and always in the same sense. The forces at work are, therefore, apparently the same in both cases; but we can only express surmises as to their nature. The rôle of salts as well as the rapid reversibility indicate that they are forces situated at the surface of the egg and the spermatozoon or both. In the first place we may think of surface tension conditions and in this respect it is possible that the entrance of the spermatozoon into the egg may be determined by such forces in a way similar to the process of phagocytosis. In the second place it may be that previous to the action of surface tension forces an alteration in the degree of fluidity of the egg surface may be required (*e. g.*, that physical change which finds its expression in the formation of the fertilization cone). Thirdly, it may be possible that before the surface tension forces can act the spermatozoon must agglutinate with the egg surface and that this agglutination is determined by certain specific substances or by certain salts ($CaCl_2$ and NaOH) or by both.

Brief mention should be made of the block discovered by Godlewski[11] to the entrance of a spermatozoon into

the egg if the sperm of the same species is mixed with the sperm or the blood of a species widely apart. If, for instance, the sperm of a sea urchin is mixed with the sperm of certain annelids (*Chætopterus*) or molluscs and if after some time the eggs of the same sea urchin are added to the mixture of the two kinds of sperm no egg is fertilized. If the solution is, however, subsequently diluted with sea water or if the egg that was in this mixture is washed in sea water, the same sperm mixture in which the egg previously remained unfertilized will now fertilize the egg. From these and similar observations Herlant[12] draws the conclusion that the block existed at the surface of the egg, inasmuch as a reaction product of the two types of sperm is formed after some time which alters the surface of the egg and thereby prevents the sperm from entering. This view is not only supported by all the experiments but also by the observation of the writer that foreign sperm or blood is able to cause after some time a real agglutination if mixed with the sperm of a sea urchin or a starfish.[13] We can imagine that the precipitate forms a film around the egg and acts as a block which can be removed mechanically by washing.

It is not impossible that the block which exists in the fertilized egg is due also to an alteration of the physical character of the surface of the egg which in this case is, however, induced from within the egg by changes caused by the entrance of the spermatozoon, which, however, are not necessarily identical with those causing development as was shown by the facts in the second chapter.

IV.

We will now turn to the question whether the motility of the spermatozoon plays no other rôle than to bring the spermatozoon so close to the surface of the egg that surface tension phenomena can engulf the spermatozoon into the egg. It is easy to show that if the spermatozoa

11 Godlewski, *Arch. f. Entwcklngsmech.*, XXXIII, 196, 1911.
12 Herlant, *Anat. Anzeiger*, XLII, 563, 1912.
13 Loeb, *Jour. Exper. Zool.*, XVII, 123, 1914.

of *purpuratus* are immobilized by NaCN no egg of the same species can be fertilized, no matter how concentrated the sperm; while the same sperm when it revives from the effect of NaCN fertilizes the same eggs at once. This meets with the possible objection that the motility of the sperm might be only necessary to allow the latter to penetrate the jelly surrounding the egg protoplasm. In order to test this objection the writer freed the eggs of *purpuratus* from this jelly by treating them for two minutes in a mixture of 50 c.c. sea water + 3 c.c. $N/10$ HCl in which all the jelly is dissolved. The eggs were washed afterwards in sea water and it was found that if sperm was added practically all were fertilized. The writer put such eggs with sperm which was immobilized by NaCN. The eggs and the sperm were squirted together with a pipette in order to bring about a close contact. No matter how concentrated the sperm was, not a single egg was ever fertilized. As soon as the spermatozoa recovered and showed only a slight degree of motility fertilization became possible. This leaves no doubt that the motility of the sperm is one of the forces required to bring the spermatozoon into the egg.

That motility is not the only force was already indicated by the previous chapter which made it clear that even if the sperm is active it can not enter the egg unless certain physical conditions at the phase boundaries of egg, spermatozoon and surrounding solution were right. In order to leave no doubt about this fact the following experiments may be quoted. If we put NaCl sperm[14] of *purpuratus* or of *Arbacia* into a neutral mixture of NaCl + KCl containing eggs of the same species the sperm will sooner or later become very active. Yet not a single egg is fertilized. If we make the solution slightly alkaline the sperm becomes at once extremely active yet with a few exceptions no egg is fertilized; while much less active sperm will fertilize all the eggs if $CaCl_2$ is added. The second fact is this: that the most active

[14] Sperm from testicles washed in $m/2$ NaCl and kept in such a solution.

sperm of *Asterias* will not fertilize the eggs of *purpuratus* in sea water while it will do so in hyperalkaline sea water (50 c.c. sea water + 0.6 c.c. $N/10$ NaOH).

We, therefore, arrive at the conclusion that aside from the physical conditions at the surface of the egg and the spermatozoon the impact of the spermatozoon against the egg is a prerequisite for the process of fertilization.

von Dungern was, as far as the writer is aware, the first to call attention to the fact that the egg itself causes resting spermatozoa to become active,[15] but curiously enough he tried to show that only foreign sperm is "stimulated" in this way by the egg (which is, as F. Lillie pointed out, not correct) and v. Dungern tried to explain on this basis why it was not possible to fertilize the egg of the sea urchin with the sperm of the starfish which had at that time not yet been accomplished.

von Dungern noticed that the egg of the sea urchin "stimulates" the spermatozoon of starfish to greater action and he concluded that since "stimulation" according to Jennings causes "motor reaction" whereby the direction of the motile organism is changed this very stimulating influence of the egg of the sea urchin upon the spermatozoon of the starfish prohibited the latter from getting into the egg. On the basis of the same idea von Dungern was consistently led to the further conclusion that the egg exercised no "stimulating" influence upon spermatozoa of its own species and that thereby the spermatozoon of the same species was enabled to get into the egg. A year after the appearance of von Dungern's paper the writer succeeded in accomplishing the hybridization of the sea urchin egg with starfish sperm by a method which contradicted von Dungern's theory, namely, by increasing the alkalinity of the sea water whereby the spermatozoon is "stimulated" to still greater activity; and on the other hand it is a common experience that a sea urchin spermatozoon becomes more active when it comes near an egg of its own species.

The writer was anxious to compare the activating

[15] v. Dungern, *Ztsch. f. allg. Physiol.*, I, 34, 1902.

action of eggs of the same and various foreign species upon spermatozoa. Since the spermatozoa of the sea urchins are usually very active in pure sea water (*i. e.*, sea water free from egg substance) it was necessary to find a solution in which these spermatozoa will keep alive for a number of days without showing any motility. Such a solution was found in a neutral $m/2$ NaCl solution and this led to the method of putting ovaries and testes directly into such solutions instead of into sea water.[16] The ovaries and testes were first washed repeatedly in these solutions to free them from the blood or its salts, and then one drop of eggs and one or more drops of the sperm suspension were mixed in a watch glass containing 5 c.c. $m/2$ NaCl (free from egg contents). In one experiment the sperm and eggs of two sea urchins, *purpuratus* and *franciscanus,* and two starfish, *Asterias ochracea* and *Asterina* (at Pacific Grove), were used. None of the four forms of spermatozoa showed any motility in a pure NaCl solution (without egg contents). In sea water (free from egg contents) the spermatozoa of the two forms of sea urchins were very active, those of the starfish were immobile. The starfish eggs were immature and did not mature during the experiment (those of *Asterias* were out of season and very small); the sea urchin eggs were mature. The result is indicated in the following table.

That there exists no strict specificity is obvious by the fact that the immature eggs of *Asterina* activate the sperm of the sea urchin *franciscanus* as powerfully as is done by the mature eggs of the sea urchin *purpuratus* and *franciscanus.* But the spermatozoa of the two species of starfish show a marked specificity inasmuch as they are activated strongly only by the (immature) eggs of their own species and only to a slight degree by the

[16] The writer had found previously that the unfertilized eggs of *purpuratus* are killed more rapidly in sea water than in a neutral $m/2$ NaCl solution, probably on account of the greater alkalinity of the former. The same may be true for the sperm of this species, athough this has not yet been tested. The unfertilized egg of *Arbacia* is more sensitive to a pure NaCl solution than that of *purpuratus.*

TABLE I

SPECIFICITY OF ACTIVATION OF SPERM BY EGGS

	Asterias ♂	*Asterina* ♂	*Franciscanus* ♂	*Purpuratus* ♂
Asterias ♀ (immature)..	*Immediately very motile.*	No activation.	Moderately active.	Slight effect in immediate contact with egg.
Asterina ♀ (immature).	Not motile.	*Violent activity.*	*Violent activity.*	Slight effect only near the egg.
Franciscanus ♀ (mature)	Slightly motile.	No motility.	*Immediately active.*	*Immediately motile.*
Purpuratus ♀ (mature).	Slightly motile after some time.	Slight effect in immediate contact with eggs.	*Immediately active.*	*Immediately active.*

eggs of the sea urchin *purpuratus*. In judging these results the reader must keep in mind first that all these experiments are made in a NaCl solution, and second, that it requires a stronger influence to activate the spermatozoa of the starfish which are at first not motile in sea water (free from egg contents) than the sea urchin spermatozoa which are from the very first very active in such sea water and which may therefore be considered as being at the threshold of activity in the pure NaCl solution.

If instead of the eggs themselves the supernatant NaCl solution from eggs is added to the sperm it is found that it requires a very much greater concentration of the supernatant NaCl solution from *Asterias* eggs to arouse the *purpuratus* sperm in NaCl into activity than if the supernatant NaCl solution from *purpuratus* or from *franciscanus* eggs is used.

The question now arises whether the relative influence of the egg on the motility of the sperm bears any relation to the power of the latter to enter the egg; or in other words if we can foretell which forms will hybridize by observing the relative activating effect of the eggs upon the spermatozoa. This does not appear to be the case on the basis of our present limited experience, since the activating effect of the *franciscanus* egg upon the sperm of *Asterias* is just as great if not greater than that of *purpuratus* eggs and yet *Asterias* sperm can enter the

latter and not the former. Even if we intensify the activity of the spermatozoon of *Asterias* by putting it in hyperalkaline sea water it will not enter the egg of *franciscanus.*

If we mix eggs of *franciscanus* and *purpuratus* in sea water and add the sperm of *purpuratus* the eggs of *purpuratus* will be fertilized more quickly than the eggs of *franciscanus;* and the reverse is true if the sperm of *franciscanus* is added to a mixture of both eggs in sea water. The writer is not quite certain that this difference is accompanied by a corresponding difference in the influence of these eggs upon the motility of their spermatozoa. It is certain, however, that the addition of egg sea water from *Asterias* does not help the fertilization of *purpuratus* eggs by *Asterias* sperm, although the egg sea water from *Asterias* increases the activity of *Asterias* sperm.

The writer is, however, of the opinion that this activating effect of the egg upon the spermatozoon is of the greatest importance for fertilization in nature and that the degree of specificity which exists (although it is far from absolute) is a means of preventing hybridization. The writer is under the impression that the eggs which are naturally fertilized in water are fertilized almost instantly after they are shed. Thus it is stated at hatcheries that the egg of the salmon loses its power of being fertilized in a few minutes and in the case of *Fundulus* the egg loses this power also very rapidly. The ripe egg of starfish dies rapidly if not fertilized. On the other hand, the writer has often been struck with the fact that the sperm of most marine forms when put into sea water is at first practically not motile. When the eggs have a specific gravity considerably greater than the water (as is the case for *Fundulus*) the eggs will sink very rapidly while the sperm remains suspended for some time. Now we have mentioned that if the absolutely inactive sperm of *Asterias* or *Asterina* comes in contact with eggs of its own species (even if they are immature) it is at once aroused into violent activity. If

the same were true for the egg of *Fundulus* fertilization could take place probably before the egg reaches the bottom of the water. If by chance a teleost of a different species would shed its sperm in the immediate neighborhood and some of it could reach the egg of *Fundulus* while it is falling the foreign sperm could probably not be aroused as quickly by the egg of *Fundulus* as the sperm of the *Fundulus* male and hence no hybridization would occur. In fish we can see that the male and female shed their sexual cells simultaneously so that they come at once in contact. The writer is inclined to believe that something similar occurs also in Echinoderms. He had last year a chance to verify once more an observation he had made for a number of years and which he had already mentioned in a previous publication.[17] The sea urchins at Pacific Grove are found in large numbers on rocks in certain coves near the shore. Up to a certain day in March every female of *purpuratus* was full of eggs. On the next day the surface of the sea in this region showed the usual indication of the spawning of large masses of animals: namely the enormous foam formation in the little coves although the sea was only moderately agitated. This foam formation is due to an increase of organic substances which lower the surface tension of the sea water and make the foam more durable. The writer realized that this might mean the end of material for some time to come and indeed not a single female of *purpuratus* of hundreds opened on that day had eggs. The condition was the same for all the sea urchins collected for two miles along the shore. During the next week immature eggs began to appear again in the sea urchins and in about ten days ripe eggs were again found. This indicates that in this region the males and females shed their eggs and sperm simultaneously. It is not impossible that among sea urchins which are found in colonies on the rocks the shedding of the sexual products of one or several individuals acts as an incentive for the whole colony. Since the eggs fall in this case also much

[17] Loeb, "The Mechanistic Conception of Life," Chicago, 1912, p. 196.

more rapidly to the bottom than the spermatozoa it is also very probable that the eggs are fertilized before they reach the bottom of the sea. We can understand under these circumstances that the specificity which exists in the activating effect of the egg upon the sperm is one of the safeguards against hybridization for eggs that are fertilized in the water, inasmuch as this specificity activates the sperm of the same species much more quickly than that of a foreign species. Other safeguards are the phase-boundary conditions which we discussed in the previous chapter.

V

If we assume that the spermatozoon bores itself into the egg by the energy of the vibrations of its flagellum it is easy to understand the importance of its motility for this process. It is, however, equally possible that a certain energy of vibration is needed to make the spermatozoon stick to the surface of the egg and that afterwards forces of a different character bring the spermatozoon into the egg. The fact that under normal conditions a very slight degree of motility on the part of the spermatozoon allows it to enter the egg seems to favor such a view.

von Dungern had already discussed the possible rôle of phenomena of sperm agglutination in fertilization as a protective agency. F. Lillie discovered the transitory agglutination of sperm induced by a substance from eggs of the same species.[18] When the sperm of the sea urchin *Arbacia* is mixed with the supernatant sea water from eggs of the same species a cluster formation occurs which may last a number of minutes and which is essentially a transitory agglutination. In *Arbacia* the agglutination is very striking, in *purpuratus* the phenomena of agglutination are not lacking but the writer was under the impression that other phenomena of the type of tropisms might enter. But he was not very certain on this point and left that question open for further discussion. The

[18] F. Lillie, *Science*, N. S., XXXVIII, 524, 1913; *Jour. Exper. Zool.*, XVI, 523, 1914.

writer is, however, under the impression that no proof for the existence of a positive chemotropism of the sea urchin sperm for the eggs of the same species has thus far been given.

The writer observed that this phenomenon of sperm agglutination depends on the motility of the sperm:[19] It only appears when the sperm is extremely motile and it lasts only a number of minutes, often only a fraction of a minute as Lillie had found. The writer observed that the duration of the clusters depended to some extent on the alkalinity of the solution. The more alkaline the latter the more rapidly the cluster scatters. The presence of a salt with a bivalent metal, especially Ca, seems necessary for the cluster formation. Sr and Ba act like Ca and so does Mg but in the latter case a slightly higher concentration is needed. The more Ca is added the more powerful the agglutination becomes. These facts suggest the following origin of the agglutination. From the jelly surrounding the eggs a certain substance is dissolved in the sea water which reacts chemically with a certain substance at the surface of the spermatozoon. If this reaction takes place in the presence of one of the salts of a bivalent metal, especially Ca, a sticky precipitate is formed on the surface of the spermatozoa, which is slowly soluble in the solution; and the more rapidly the more alkaline the solution. If the spermatozoa are very active the impact with which they strike each other may lead to their sticking together and this agglutination will last until the precipitate is dissolved again.[20]

The writer mentions this fact here because it might give us a clue to the rôle of the motility of the spermatozoon for its entrance into the egg. One can imagine that the spermatozoon must stick to the surface of the egg in order to be taken into it and this sticking may not come about unless the spermatozoon strikes the surface of the

[19] Loeb, *Jour. Exper. Zool.*, XVII, 123, 1914.

[20] Lillie measures the degree of agglutination by its duration; if our assumption is correct he really measures the time required for the solution of the sticky precipitate on the surface of the spermatozoon by the sea water.

egg with a certain velocity. This is, however, merely a suggestion. The really serious difficulty of such an assumption lies in the fact that the specific and transitory cluster formation or agglutination of the spermatozoa is not a general phenomenon. It may even turn out to be confined to sea urchins and certain annelids. It is probably lacking in all cases of hybridization. Yet this would not necessarily speak against the possibility of an agglutination of the spermatozoon to the egg as a prerequisite of fertilization.

This latter idea receives some support in the writer's experiments on heterogeneous hybridization. He was able to show that both NaOH as well as $CaCl_2$, which render possible the fertilization of the eggs of certain sea urchins through the sperm of starfish, also favor the agglutination of that sperm to the chorion of the egg. This leads to the peculiar phenomenon of mere membrane formation in the egg by the living spermatozoon without the entrance of the latter into the egg.[21]

VI

Lillie seems to take it for granted that the substance of the egg which causes sperm agglutination is identical with the substance which stimulates the spermatozoa into greater activity. If this were correct the conditions for the two phenomena should be identical, which is however far from being the case.

The writer showed that if we deprive the eggs of *purpuratus* of the jelly which surrounds them and if we wash them afterwards a few times in sea water to deprive them of the last vestiges of jelly substance which may still adhere to them they have lost completely and permanently the power of forming clusters with the sperm of their own species. Such eggs were washed four times in $m/2$ NaCl and when a drop of the supernatant NaCl solution was added to NaCl sperm of *purpuratus* which was not motile it activated the sperm very powerfully.

The writer had found that the egg sea water of *S. fran-*

[21] Loeb, *Arch. f. Entwcklngsmech.*, XL, 310, 1914.

ciscanus does not give a trace of agglutination with the sperm of *purpuratus* but if the experiment is made in $m/2$ NaCl solutions it can be shown that the *franciscanus* egg NaCl solution activates the NaCl sperm of *purpuratus* in an $m/2$ NaCl solution very strikingly.

The immature eggs of *Asterias ochracea* activate the otherwise non-motile sperm of the same species, but the eggs of this starfish do not give any agglutination reaction with their own sperm and Lillie found the same for the starfish in Woods Hole. It might be said that all this only proves that the activating effect requires a smaller concentration than the agglutinating effect, but may yet be caused by the same substance. This objection is, however, not tenable in the following case.

Purpuratus sperm washed in $m/2$ NaCl is as a rule more active in a mixture of NaCl + KCl than in a mixture of NaCl + $CaCl_2$ (if both solutions are free from egg contents); yet in the latter solution the agglutination reaction upon the addition of egg-NaCl is very strong while in the former it is lacking (unless the sperm or testicles or ovaries give off some $CaCl_2$ to the surrounding solution). Again it might be argued that the activation of the spermatozoon might be induced by the same substance as the agglutination, but that the agglutinating substance in both cases reacted with different constituents of the spermatozoon. While this may be admitted, it must also be conceded that with the facts which we have at our disposal at present we can not be certain that the aggultinating and activating substances are identical.

VII

Lillie[22] not only takes the identity of the two substances for granted but he assumes that without the agglutinating substance in the egg (to which he gives the somewhat prejudicial name "fertilizin") no fertilization is possible. Fertilization in his opinion consists in the combination of the spermatozoon with a molecule of "fertilizin" in

[22] *Loc. cit.*

the egg, whereby the fertilizin molecule undergoes a change in the other end and this change causes the egg to develop. The fertilizin is thus an "amboceptor" in the sense of Ehrlich's side-chain theory.

The side-chain theory was invented by Ehrlich for an altogether different purpose. Bordet had found that for certain phenomena of immunity two substances were needed (which Ehrlich named amboceptor and complement, respectively). Ehrlich assumed that they were bound chemically by the antigen (the substance against which the organism was immunized) but found that while the antigen (A) was able to bind B (the amboceptor) in the absence of C, it was not able to bind the complement C in the absence of B. From this Ehrlich concluded that of the two possible modes of linkage between the three bodies $A{<}^{B}_{C}$ and $A—B—C$ the latter was the one which really occurred. Since in this case C is not directly linked with A but through the intermediation of B he called B the "amboceptor" and the scheme of linkage a "side-chain" linkage.

Lillie applies this theory (which covers the two possible modes of linkage of two chemical compounds to a third one) to the entrance of the spermatozoon into the egg, by calling the egg an antigen A and the spermatozoon a complement C and assuming the existence of a hypothetical amboceptor B in the form of the substance that causes agglutination, the "fertilizin." Even if we are willing to overlook the fact that the egg and the spermatozoon are cells and not simple organic compounds and if we are willing to overlook the further fact that the assumption of an amboceptor as a connecting link between the two is arbitrary we can not overlook the fact that the spermatozoon does not combine chemically with the egg but that it actually enters into the egg and attaches itself to the egg nucleus. It seems then futile to discuss whether the spermatozoon combines with the egg in side-chain fashion (namely, Egg—Fertilizin—Spermatozoon) or in direct

fashion, namely,

$$\text{Egg}\left\langle\begin{array}{l}\text{Fertilizin}\\ \text{Spermatozoon}\end{array}\right.$$

since the engulfing of the spermatozoon into the egg is a physical process which bears no relation to either possibility.

It has been stated that the "fertilizin theory" explains also the phenomena of artificial parthenogenesis just as well as any other theory. In a recent book on artificial parthenogenesis the writer has given the results of a large number of experiments and he has tried to explain some of them; the reader would, however, vainly look for a "theory" of artificial parthenogenesis. A theory in a scientific sense consists in the presentation in mathematical or numerical form of a phenomenon as the function of its variables. The writer has tried to prepare the ground for such a treatment of the phenomena of fertilization and of the first development of the egg by working out those variables which permit a quantitative treatment, but even if the exploration had been advanced further than it actually has been, it would not be possible to ever expect that a single theory could cover all the phenomena of fertilization and development, since under these two headings so many physically and chemically different processes are included (of which one follows the other) that they can not be covered by one theory. It is true the writer had in former publications occasionally used the term "lysin theory of fertilization" but only to express the fact that cytolytic agencies induce membrane formation and that the membrane formation induced by a spermatozoon might also be due to a cytolytic agency contained in the spermatozoon; but he has dropped this term in his recent book on the subject.

While the writer does not desire to enter into a further discussion of the side-chain theory of fertilization he wishes to point out that it rests on the claim that that substance which causes sperm agglutination is contained

in the unfertilized egg and that the egg can only be fertilized as long as this "fertilizin" is present in the egg. It is obvious that such an assumption demands for its proof that in all cases in which an egg can be fertilized it must contain the agglutinating substance. There is only one test for the presence of this substance, namely the cluster formation of the sperm in the presence of egg sea water. This proof can not be furnished since, as the writer had shown in a former paper, the reaction is lacking in many cases of hybridization; it is also lacking in the case of the starfish.[23] It is not impossible that if the theory is tested further it will be found lacking in a considerable number of cases. To this objection Lillie replies that it is not necessary that the eggs should actually give the agglutinin reaction, it is sufficient that the agglutinating substance is contained in the egg. But how can we tell that it is contained in an egg which fails to give the agglutination reaction as long as this reaction is the only reliable test for the presence of the agglutinating substance in the egg? Rigorously speaking, even if all eggs of every species gave the agglutinin reaction it would still be necessary to furnish a direct proof that the agglutinin has anything to do with fertilization and development.

It may be possible that Lillie considers such a proof to be contained in the following statement.

> I adopted then the working hypothesis that this substance[24] is necessary for fertilization and there followed immediately three corollaries, viz.: (1) if it were possible to extract this substance from eggs they would no longer be capable of fertilization; (2) fertilized eggs are incapable of uniting again with spermatozoa, hence if the hypothesis is correct they could no longer contain free fertilizin; (3) eggs in which membranes have been formed by methods of artificial parthenogenesis become incapable of fertilization; such eggs must also therefore be devoid of free fertilizin after they have reached the non-fertilizable condition if the hypothesis is correct. These consequences were actually found to be true.[25]

[23] Lillie, *Biol. Bull.*, XXVIII, 18, 1915.

[24] The "fertilizin."

[25] Lillie, *Jour. of Exper. Zool.*, XVI, 523, 1914.

Of these three "corollaries" the first one is the most important, since it claims that the power of the eggs of being fertilized varies with their contents of fertilizin. The proof consisted in this: that eggs were washed a number of times during three consecutive days and after two days the percentage of eggs that could be fertilized were diminished to about one third.

> There is thus the anticipated decrease in the percentage of fertilizations.

It is a well known fact that the unfertilized eggs of the sea urchin (in fact of all marine animals) perish when they lie for some time in sea water and one of the main causes of this phenomenon is also known, namely oxidations. If the oxidations are inhibited through the removal of oxygen or the addition of KCN the life of the eggs can be prolonged.[26] In the mature starfish egg this death which is accelerated by the temperature (and has the high temperature coefficient of many life phenomena) takes place in a few hours,[27] while it begins a little later in the egg of the sea urchin. After the artificial membrane formation it takes place very rapidly also in the sea urchin egg (coincident with the enormous increase in the rate of oxidations caused by the artificial membrane formation) and in this case the death of the egg can also be retarded by the withdrawal of oxygen or the addition of cyanide.[28] In view of these facts the objection can not be avoided that in Lillie's experiment the number of eggs which could be fertilized fell off after two days to one third not on account of the loss of "fertilizin" but because of the fact that two thirds of the eggs were dead by that time. That this assumption is well grounded is testified by Lillie's own remarks:

> Concomitantly, with these effects of the series of washings the developmental energy becomes greatly reduced. This was very obvious from the second fertilization.[29] On August 24 (48 hours after fertilization) a large quantity of living material was contained in the second *A* fertili-

[26] Loeb and Lewis, *Am. Jour. Physiol.*, VI, 305, 1902.

[27] Loeb, *Biol. Bull.*, III, 295, 1902.

[28] Loeb, "Artificial Parthenogenesis and Fertilization."

[29] Which occurred on the second day.

zation but none had even approximately pluteus structure. The most common form was a stereoblastula. In the second *B* fertilization there were a few abnormal prismatic plutei, while the majority were gastrulae. The third fertilization resulted in extremely abnormal ciliated types. The fourth and fifth did not proceed beyond abnormal cleavage stages. From this and similar experiments Lillie draws the following conclusion:

The eggs have evidently lost something which affects their power of fertilization. Table 3 shows the measure of loss of the sperm agglutinating substance and justifies the general conclusion that this is a factor in the result. The loss of other substances may also combine in the decrease of fertilizing power, but of this we know nothing definite. As a matter of fact, fertilizing power is gradually lost with decrease of fertilizin content of the egg.

It seems to the writer that in these experiments the power of being fertilized was gradually lost by the death of the eggs. And an additional justification of this criticism is given by the following fact, that if we deprive *fresh* eggs of *purpuratus* permanently of their power of giving off "fertilizin" their power of being fertilized is not only not lost but is entirely unaltered. The writer has shown that if the eggs of *purpuratus* are treated for two or three minutes with a mixture of 50 c.c. of sea water + 3 c.c. of HCl (whereby the jelly surrounding the egg is dissolved) and if the eggs are washed they give no trace of a fertilizin reaction but 100 per cent. of the eggs can be fertilized.[30]

It might be argued that the supernatant sea water from these eggs had not lost all power of causing agglutination of the sperm. This the writer must deny but for arguments' sake he will admit that a trace near the "psychological limit" might have been overlooked where a "fertilizin" partisan might have declared that he still could perceive a faint indication of a "fertilizin" reaction. In that case only a few eggs should have been fertilized—the fertilizin theory rests on this assumption; in reality, however, practically one hundred per cent. were fertilized in every case (provided the eggs had not been lying in the see water too long, *i. e.*, more than a day or two).

[30] Loeb, *Jour. Exper. Zool.*, XVII, 123, 1914.

To this Lillie replies that perhaps the sperm of *purpuratus* is not so delicate an indicator for agglutinin as the sperm of *Arbacia*—but as long as the agglutination reaction is the only test for the presence of fertilizin in the egg, such an answer begs the question.

From the fact that the power of agglutinating the sperm is lost if the egg of *purpuratus* is deprived of its jelly by acid treatment the writer drew the conclusion that in this egg the "fertilizin" does not come from the unfertilized egg but only from its jelly and that this was contrary to Lillie's assumption. To this Lillie[31] replied by pointing out that the immature eggs of *Arbacia* do not give the agglutination reaction while the mature *Arbacia* egg gives the reaction very powerfully, and that we must conclude from this that the "fertilizin" contained in the jelly comes from the egg and is given off during the period of the maturation divisions (the latter statement, however, is after all only an assumption though a probable one). But this does not meet the question at issue, namely that in the egg of *purpuratus* at the time of maturity the fertilizin which is given off is contained exclusively in the jelly and not in the egg, as it should be if the presence of fertilizin in the egg were a prerequisite for its ability of being fertilized. It is true that if we repeat this experiment in the egg of *Arbacia* we find that after the removal of the jelly by HCl a trace of the agglutinating substance may still be given off by the egg, although little in comparison with that given off by the jelly. But this does not alter the facts as they are found in the egg of *purpuratus*.

As far as the two other proofs of Lillie are concerned, we have already touched upon them in the previous parts of this paper. The fact that the fertilized eggs of *Arbacia* (and of *purpuratus*) cease to give the agglutinin reaction is due to the loss of the jelly on the part of the fertilized egg to which in *Arbacia* should be added the fact that some of the material of the cortical layer is given

[31] Lillie, *Biol. Bull.*, XXVIII, 18, 1915.

off during the process of membrane formation. The writer has pointed out in former papers that the cortical layer of the egg which undergoes liquefaction in the process of membrane formation behaves towards reagents very much like the jelly which surrounds the egg.[32] But since in the egg of *purpuratus* the loss of this agglutinating power on the part of the egg is not necessarily accompanied by the loss of the power of being fertilized—*e. g.*, in the HCl experiment—we are inclined to believe that there must be another reason that an egg fertilized by sperm can not be fertilized a second time.

As far as the statement is concerned that the egg can no longer be fertilized after artificial membrane formation by butyric acid the writer can not admit the correctness of this statement (see Chapter III). In the eggs in which artificial membrane formation has been called forth by butyric acid the main if not the only block to a subsequent fertilization is the membrane itself.

This can be proved by a very simple experiment. If we call forth the membrane formation in the egg of *purpuratus* in a neutral or faintly alkaline solution of $m/2$ ($NaCl + KCl + CaCl_2$) (instead of in sea water) a very thin membrane is formed, which is easily torn and offers no resistance to the spermatozoon. All the eggs treated in this way can be fertilized by sperm. The agglutinin reaction of such eggs is, however, permanently lost.

The facts thus far known seem to force us to the conclusion that no adequate proof has been offered thus far for the connection between the power of an egg of being fertilized by sperm and its power of causing a cluster formation of the sperm. The writer has pointed out in a previous paper that it is difficult to see why there should exist such a relation, since sperm agglutination can only inhibit the entrance of the spermatozoon into the egg.

32 "Artificial Parthenogenesis and Fertilization," Chicago, 1913, pp. 210–14. University of California publication, Physiology, Vol. 3, p. 1, 1905.